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# Morphometric analysis in ethnic neonates from multiple substance exposure

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## 1. ABSTRACT

In the United States, approximately 10% of newborn infants are exposed prenatally to alcohol and/or illicit substances. However, no studies have evaluated the compounding effects of multiple illicit substances exposure *in utero* as potential teratogen (s). The potential teratogenic effects of nicotine and illicit substances (e.g. cocaine, marijuana and heroin) have previously been studied but there has been no documentation of facial landmark dislocation (s). Our goal is to investigate whether morphometric analysis could differentiate facial landmark dislocations in neonates of African descent, when exposed to alcohol, nicotine and illicit substances, either singly or in combination. Craniofacial features from a cohort of 493 African-American neonates less than 48 hours of age were analyzed by Multivariate Hotelling's $T^2$ analysis of 99 relevant facial landmark triangles. Morphometric analysis discriminated unique asymmetries in groups of certain illicit exposure(s). Neonates with multiple prenatal exposures had fewer facial landmark dislocation(s) compared to single exposures. Deviation from normal facial features has the potential to be used as a screening tool for prenatal exposure to some illicit substances.

## 2. INTRODUCTION

The National Institute of Drug Abuse (NIDA) has warned that substance abuse is a serious health concern. There are published reports indicating that in 1999, the lifetime illicit substance use among females between 18 - 35 years of age ranged from 41 to 45% (1). In 1998, self-reporting for specific usage of substances in African-American females using marijuana (23.5.%), cocaine (5.6.%) (when used in the form of crack, 2.7.%) and psychotherapeutics (5.5.%) were noted (2). In 2002, the lifetime usage reported by African American woman of childbearing age (19 - 35 years) for marijuana (31%), cocaine (8.6.%) and crack cocaine (3.4.%) were additionally reported. Also, 28.6.% of African-American adolescent youth (ages 12 - 17 years) reported using illicit substances at some point in their lifetime (3).

The devastation of substance use and abuse not only affects the pregnant mother but also her developing and established family and community at large. Despite continuous efforts to curtail the substance abuse problem in this nation, our vulnerable population is exposed *in utero*. The use of alcohol as a common substance of abuse is often associated with other substances of abuse (3). This leads to difficulty in teasing out the effects of exclusive alcohol exposure from the effects of other substances on the development of facial features and that of the central nervous system in the fetus. Neonates born to alcohol abusing mothers have long been studied in the context of fetal alcohol spectrum disorders (FASD), which is characterized by a combination of facial anomalies, growth retardation, and cognitive and neurobehavioral disorders.

By definition, a teratogen is any substance that alters the structure or function of the developing fetus (4)_ENREF_1. The main elements which contribute to altered development include: (1) *timing of the exposure*: this essence relates to majority of the congenital anomalies when exposure is during the 3$^{rd}$ to 8$^{th}$ week of gestation. Whereas, exposure during the latter part of pregnancy leads to intrauterine growth retardation (IUGR). However, the developing central nervous system is affected from exposure during any part of pregnancy. (2) *bioregulation of the placenta* – this includes the active regulation of oxidation, reduction, hydrolysis and conjugation of substances that enables them to cross the placental barrier (4). A drug capable of crossing the blood-brain barrier of the mother can also cross the placenta barrier and affect the developing nervous system (4); and (3) *bioavailability of substances* - the potential teratogenicity of a substance is not only dependent on its quantity but also on its pharmacokinetics, pharmacodynamics and pharmacogenomics regulation (s). This can be determined by the genotype of the pregnant mother and her fetus. In addition, not every fetus will be affected by these exposures; for example, only ~6% of babies exposed to alcohol in the critical period are clinically identified to have the characteristic dysmorphic facial features found in FASD (5).

Alcohol exposure in the first two months of gestation has been shown to alter neural crest cell migrations, causing various minor facial anomalies (6-8). Face landmark represents points of interest on face and *facial landmark dislocations* detect deviation from normal landmarks. The use of morphometrics to delineate *facial landmark dislocations* is an established tool that identifies craniofacial dysmorphism in FASD. It has also been used to screen neonates at risk for abnormal brain development and long term cognitive deficits (9, 10)_ENREF_6. Although phenotypic variability in facial facies of FASD (short palpebral fissures, elongated upper lip, deficient philtrum) (8) can be clinically visible, there is difficulty correlating the quantity of alcohol consumption to FASD due to variabilities in at-risk modifiers (11). There have been reports of other antenatal substances use affecting cranial facial and/or skeletal dysmorphism; however, they have not been characterized for deviation in facial landmarks. Examples of such substances are: (1) antenatal use of anticonvulsant valproic acid, in the first trimester of pregnancy, can manifest a well-recognized cluster of facial dysmorphism, congenital anomalies and neurodevelopmental retardation of "fetal valproate syndrome". Many of these present as craniofacial dysmorphisms (prominent metopic sutures, trigonocephaly, tall forehead, epicanthal folds, infraorbital groove, and medial deficiency of eyebrows, shallow philtrum, anteverted nares, and broad root of nose, low set ears, thin upper lip, and small mouth) (12); and (2) reports of *in utero* exposure to marijuana, cocaine, or lysergic acid diethylamide (LSD) have shown that neonates manifest a variety of congenital abnormalities including IUGR and limb defects (13-15). The use of *facial landmark dislocations* could be as a new territory as a potential screening tool for neonates at risk for abnormal brain development and long term cognitive deficits when exposed to illicit substances antenatally, either individually or in combination.

### 2.1. Pathophysiology of common and illicit substances

Almost all drugs are known to cross the placenta and have some effect on the fetus. They have prolonged and higher concentration on the fetal end (16). The pathophysiologic effect of illicit substances use in pregnancy involves variable degree (s) of damage to the developing central nervous system of the fetus (17-19). A commonly studied teratogen, alcohol, induces a direct toxic effect on cells mediated by a hypoxic environment that alters cell migration and programmed neuronal death. The vulnerability of the fetal brain to alcohol-induced neuronal damage has been associated with the loss of Purkinje cells (20), retina ganglions and eye malformations (21, 22). Studies have indicated lowered neuropsychological and intellectual performance in children (23).

The pharmacokinetics of other commonly abused substances may offer insight into possible mechanisms of substances-induced damage of the developing fetal brain. Nicotine crosses the placenta barrier and concentrates higher in the fetal compartment. The exact mechanism of nicotine teratogenicity is unknown. Studies have shown that it produces a hypoxic (mediated by cyanide and cadmium toxicity as well as reduced uterine blood flow) and undernourished environment, resulting in IUGR; It alters brain metabolism and neurotransmitter systems with significant deleterious effects on brain development (16). Cocaine, another common substance of abuse, has a small molecular size that enables ready

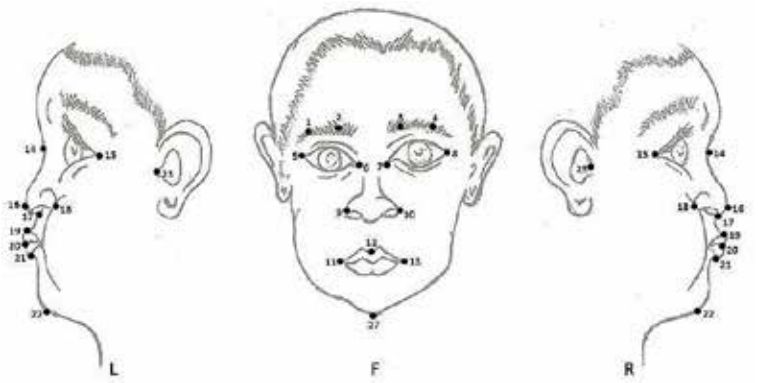


**Figure 1.** Facial sketches illustrating the 24 facial landmarks selected for this study. The numbered points represent the most reliably identifiable facial landmarks from photographs used by clinicians.

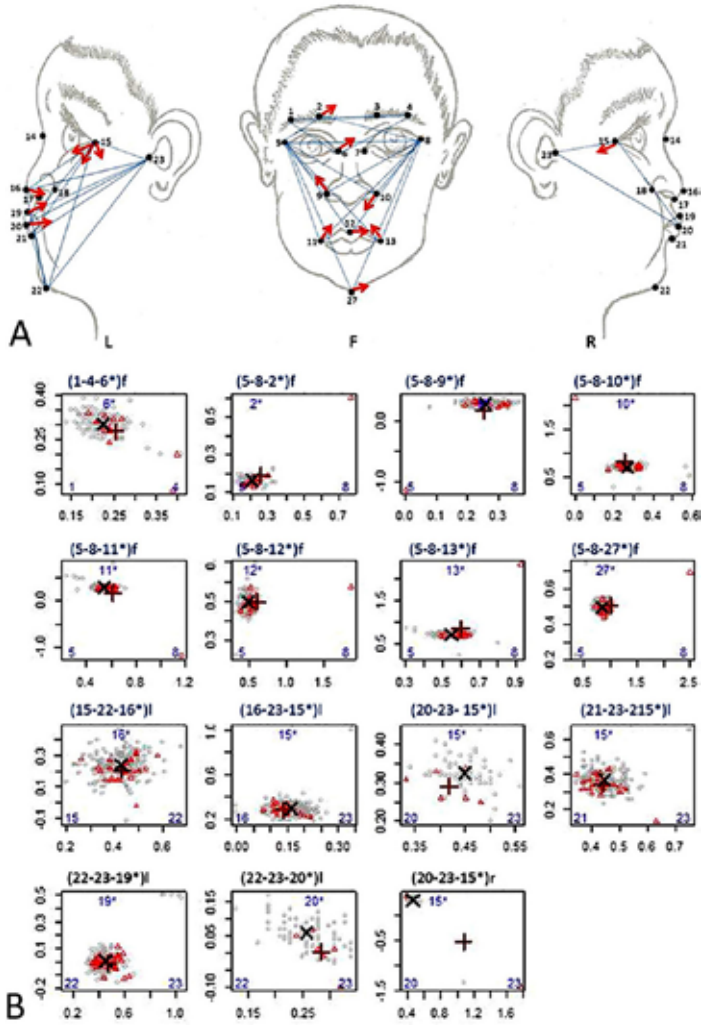


**Figure 2.** Facial landmark deviations in neonates exposed to Alcohol only. A) Red arrows represent directional vector changes with respect to facial landmarks and clinically relevant triangles (L, left laterals; F, frontals, R right laterals). This denotation is applied to all facial landmark deviation figures in this document. Eight frontal, one right and seven left lateral triangles were significantly altered (p≤0.0.5). B) Hotelling's $T^2$ test discrimination of statistically significant triangle deviations contrasting alcohol exposed and control group. Each scatterplot is scaled to denote the triangle and its three vertexes. Grey circles represent controls and red triangles represent exposed neonates. The black 'X' denotes the averaged center for controls, while the red '+' denotes the averaged center for exposed group. Net vector changes were observed in accordance with the X and Y-axis. This denotation will be applied to all Hotelling's $T^2$ tests discrimination figures in this document.

**Table 1.** Neonatal exposure to substances and classification of groups

| | | |
|---|---|---|
| **Number of neonates exposed to the teratogen (%)** | Alcohol | 84 (46.7.) |
| | Nicotine | 131 (72.8.) |
| | Marijuana | 44 (24.4.) |
| | Cocaine | 42 (23.3.) |
| | Heroin | 7 (3.9.) |
| **Classification into seven teratogen groups (%)** | *Single Exposures* | |
| | (1) Alcohol only | 29 (16.1.) |
| | (2) Nicotine only | 56 (31.1.) |
| | (3) Substance only | 12 (6.7.) |
| | *Multiple Exposures* | |
| | (4) Alcohol + nicotine | 21 (11.7.) |
| | (5) Alcohol + substances | 8 (4.4.) |
| | (6) Nicotine + substances | 28 (15.6.) |
| | (7) Alcohol + nicotine + substances | 26 (14.4.) |

Total subjects=493, Control (unexposed) neonates (%) 313 (63.5.), Exposed neonates (%)180 (36.5.)

crossing of the placenta barrier and concentration in the amniotic fluid at higher concentration to that of maternal serum (24). Cocaine blocks norepinephrine uptake in sympathetic neurons and in the uterine muscle. Inhibition of neuronal norepinephrine uptake leads to increased sympathetic activity, resulting in cardiac stimulation, vasoconstriction and consequent hypertension. Significant hypertension decreases blood flow, transfer of essential nutrients and oxygen from mother to fetus, causing fetal hypoxia, intrauterine growth retardation (IUGR) and premature delivery (25). Studies in humans and animals showed that the hypoxic teratogenicity of cocaine creates reactive-oxygen species (ROS) causing genotoxicity, neurotoxicity and mutagenesis (19). Prenatal exposure to opiates has a detrimental effect on prenatal growth but there are conflicting evidence on its effect on brain development (16) (26).

Unlike other drugs, fetal exposure to marijuana is limited by the placenta. Although fetal δ-9-tetrahydrocannabinol (THC) concentrations is lower than that of the mother (16), its remains in circulation for up to 30 days resulting in prolonged exposure, causing on prenatal effects (16, 27, 28). Smoking marijuana produces as much as 5 times the amount of carbon monoxide as does cigarette smoking, perhaps altering fetal oxygenation (29).

### 2.2. Applications of Morphometric research

Clinical evaluations for alcohol exposure are routinely used to screen for facial and other physical anomalies. This evaluation relies on the skills, experience and judgment of clinicians. There have been reports of considerable inter-observer variability when minor anomalies are evident (30). Anthropometry, the science of measuring an individual's physical form and shape, has been applied to the study of facial morphometrics as an aid to detect dysmorphism. This method is relatively simple, inexpensive, and noninvasive (31), (32), and (33). The developments in, and applications of, geometric anthropometry has evolved into the science of morphometric analysis in syndrome delineation (34-37). Initial studies that identified congenital anomalies of FASD used facial shapes with relative positions of important facial landmarks to characterize clinical manifestations, comparing the mean shapes of triangles between exposed and unexposed groups (38, 39). Advances in traditional anthropometry were developed to study facial landmarks in two-dimensional (2D) photogrammetry (40) and subsequently three-dimensional (3D) stereo-photogrammetry (41, 42). However, Geometric Morphometrics (43) accounts for superimposition methods of the Generalized Procrustes analysis, eliminating non-shape variations in configurations of landmarks. This method overlays shapes based on centroid (origin of each configuration) in a common (arbitrary) system, then scales to a common unit size (by dividing by the centroid size (34, 35) ). The variation in the configuration size with respect to the axes can be treated as multivariate data. Mutsvangwa's data validated the reliability and precision of direct and stereo-photogrammetric measurements. His data, using dolls, showed 100% reliability within a 1.5. mm error range (92.4.% were within 1mm range), with precision in >99% of measurements and 72.2.5% of measured distances were within 1mm range for measurement error (44, 45). Bookstein's coordinate system for sliding superimposition procedure optimally translates, scales and rotates landmarks for coordinate points with association for the centroid (34).

Previous data using facial image analysis has indicated that variation in the pattern or degree of dysmorphism is due to clinician inaccuracy and photograph non-reliability (44, 46, 47). However, utilizing Bookstein's system, two new coordinates on the x- and y- axes can be combined to compare the significance of shape dislocations of well-established facial landmarks between test and control groups using the Multivariate analysis of Variance (MANOVA) based on Hotelling's $T^2$ statistic improved accuracy and reliability (48). The use of

this system can avoid the high costs associated with 3D photogrammetry and scanning. In large-scale screening for teratogenic effects, stereo-photomorphometry is commonly used as a morphometric tool for detecting *facial landmark dislocations* for clinical evaluations of FASD (41, 44, 49-55).

Other than alcohol, we sought to establish whether morphometric analysis could differentiate facial dysmorphism associated with substance exposures and whether these exposures differ in their capacity to induce distinguishable facial dysmorphisms. This application is novel in that no studies have been conducted to differentiate facial landmarks in African-American neonates exposed to alcohol, nicotine and some illicit substances. Nicotine and illicit substances (*e.g.* cocaine, marijuana and heroin) have been well studied for their teratogenic potential with no documented evidence of *facial landmark dislocations*. Therefore, the intent of this study is to evaluate the potential for morphometric analysis to detect teratogenic effects of alcohol, nicotine and other illicit substances (cocaine, marijuana and heroin) in exposed neonates and the potential to serve as an early predictor of neurological deficits. We hypothesized that the power of morphometric analysis would detect minor abnormalities of facial structures (that may not be visible to the eyes of the clinician) from a combination of singular and multiple exposures during periods of fetal development.

We obtained morphometric data from neonates less than 48 hours of age as a novel approach aimed to detect facial dysmorphism in African-American neonates before maturation-induced minimization occur. This phenomenon is defined by basicranial structure change in a spatio-temporal ontogenetic cascade over time which reduces distinguishable features due to maturation of the cranium development.

## 3. MATERIALS AND METHODS

### 3.1. Subjects

A randomly selected cohort of 517 African-American mothers who delivered at Howard University Hospital and DC General Hospital in Washington DC were administered screening questionnaires (with a written consent) by the project coordinator. This study was approved by Institutional Review Board (IRB) at Howard University. Mothers provided demographic information, family history, prenatal and medical history, alcohol, nicotine and other substances (*i.e.* cocaine, marijuana and heroin) consumption per recall. Enrollment in this study was conducted following birth of the neonate; therefore, quantification of substances abused was not categorically documented. Within 48 hours after birth, the neonates' facial features were photographed adhering to previously established photogrammetry methods (56). Neonates born with genetic disorders or those for whom complete clinical and medical information were not obtained were excluded from this study. After exclusion, 493 neonates were enrolled in the study. Three hundred thirteen neonates were not reported to be exposed to any substance, these neonates served as a control group. The remaining 180 neonates were reported to have gestational exposure as follows: alcohol (84), nicotine (131), marijuana (44), cocaine (42) and heroin (7). There were some pregnant mothers who consumed more than one substance and therefore their neonates were re-classified based on teratogenic exposure groups, described below.

### 3.2. Study Design

Our goal was to investigate whether morphometrics could determine which features, if any, best differentiated craniofacial changes induced by alcohol, nicotine and other substance exposure during prenatal development. We hypothesized that morphometric analysis would differentiate *facial landmark dislocations* in neonates with single or combined exposure. Several facial photographs of frontal, left and right laterals were taken within 48 hours after delivery while maintaining standards of direct photogrammetry (57). There was a focus on those clinically relevant landmarks known to delineate fetal alcohol syndrome (s) and remain unaltered post organogenesis (38), definitions of facial landmarks are shown in Table 2.

### 3.3. Morphometric and statistical analysis

Construction of a facial map and analysis was performed as previously established by Bookstein (56, 58-62). Interval measurements were calculated between the 24 landmarks (in raw scale), then standardized by scaling down on a 0 to 1 scale relative to the base of that triangle when applying Bookstein's x- and y- coordinates, thereby establishing a total of 99 facial triangles (29 frontal, 35 left and 35 right). A new Cartesian coordinate (X-, Y-) was mathematically quantified with respect to the longest dimension of the triangle, defined as the base. Certain landmarks on the face are known to shift as various contours of the face present variant features. Therefore, to standardize for size and shape irregularities, the centroid was characterized with respect to the triangle. The means were calculated using the two-sided t-tests (for all x-coordinates and all y-coordinates separately) assuming unequal variances with significance level of $p \leq 0.0.5$ (not shown) to contrast between the unexposed and exposed groups. Analysis of equal variances was also performed along with unequal variances, but numbers remained similar within a few decimal points (not shown). Utilizing the extended applications

**Table 2.** Definitions of anatomical landmarks in photographs

| Landmark | Name/Definition |
|---|---|
| ***Frontals*** | |
| 1,4 | Frontotemporal - Intersection of the eyebrow curve and a vertical line through the exocanthion |
| 2,3 | Intersection of the eyebrow curve and a vertical line through the midpoint of the palpebral fissure/superious |
| 5 | Left outer Canthus |
| 6 | Left inner canthus |
| 7 | Right inner canthus |
| 8 | Right outer canthus |
| 5,8 | Exocanthion - Lateral intersection of upper and lower eyelids at the outer canthions |
| 6,7 | Entrocanthion - Medial intersection of upper and lower eyelids at the inner canthions |
| 9,10 | Superior Alare - Alare curvature formed by the left and right alare |
| 11,13 | Chelion - Lateral intersection of upper and lower vermilion. |
| 12 | Labiale Superius - Midpoint of upper vermilion border |
| 27 | Lowest tip of the chin |
| ***Laterals*** | |
| 14 | Nasion - Point of maximum curvature over nasal bridge |
| 15 | Exocanthion - Lateral intersection of upper and lower eyelids |
| 16 | Pronasale - Point of maximum curvature over nasal tip |
| 17 | Subnasale - Intersection of columela and philtrum |
| 18 | Point of maximum curvature of soft tissue fold from zygoma |
| 19 | Labiale superious - Border of upper vermilion and philtrum |
| 20 | Stomion - Lateral intersection of upper and lower vermilion. |
| 21 | Labiale inferious - Border of lower vermilion and lower lip |
| 22 | Gnathion - Point of maximum curvature of chin |
| 23 | Tragnion - External auditory opening |

Multivariate Analysis of Variance (MANOVA) of Hotelling's $T^2$ statistic, the coordinates were treated as multivariate variables, and dislocations of facial triangles with respect to the centroid were compared. Statistical analysis was performed using JMP, SPSS and/or R software.

## 4. RESULTS

The investigation of illicit substance consumption of mothers, singly or in combination leads to specific exposure-induced facial landmark changes. Statistically significant triangles are documented in Table 3. Each Hotelling's scatterplot was scaled to denote a triangle with three vertices, where the greatest length is defined as the base and denoted as the X-axis. Grey circles represent control cases and red triangles represent substance-exposed neonates. The large black X-symbol in the scatterplot denotes the averaged center for controls, whereas the large red + symbol denotes the averaged center for the exposed group. The net change in facial structure is represented by directional vector changes in accordance with the X and Y-axis and corresponding Figures of faces with respect to the base of specific triangles. These denotations were applied to all Hotelling's $T^2$ test scatterplot Figures and were further used to explain the results of facial structure changes.

### 4.1. Craniofacial dysmorphism related to substance exposure

#### 4.1.1. Alcohol exposure

African-American neonates exposed to only alcohol showed significant landmark dislocations for eight frontal triangles, one right and seven left lateral triangles. Observations of frontal landmark displacements suggest reduced entrocanthion distance, reduced interpupillary distance, lowered nasal bridge, increased superior alare curvature resulting in an upturned nose, lateral dislocation in the chelion, and smaller or indistinct gnathion. The dislocations represented by the left and right lateral triangles showed increased exocanthion distance and downwardly slanting eyes, heightened prosonale, indistinct labiale superious, and reduced cheilion and stomion. These results are consistent with previous data indicating that exposed children have rum defects such as small faces, short palpebral fissures, and a hypoplastic midface (40, 50). In this study, African-American neonates exposed to alcohol displayed variable expression of these anomalies.

#### 4.1.2. Nicotine Exposure

In African-American neonates exposed to only nicotine (Figure 3), the dislocation of the frontal

**Table 3.** Hotelling's $T^2$ analysis of statistically significant altered facial landmark triangles by exposure groups

| Facial landmarks | Control (n) | Test (n) | F statistic | P value |
|---|---|---|---|---|
| **SINGLE EXPOSURES** | | | | |
| **Alcohol only** | | | | |
| (1-4-6*)f | 159 | 13 | 4.4.922 | 0.0.126 |
| (5-8-2*)f | 158 | 12 | 4.6.963 | 0.0.104 |
| (5-8-9*)f | 141 | 13 | 5.8.956 | 0.0.034 |
| (5-8-10*)f | 160 | 13 | 5.2.063 | 0.0.064 |
| (5-8-11*)f | 158 | 13 | 6.7.327 | 0.0.015 |
| (5-8-12*)f | 162 | 13 | 7.3.627 | 0.0.009 |
| (5-8-13*)f | 158 | 13 | 6.8.098 | 0.0.014 |
| (5-8-27*)f | 149 | 13 | 5.7.023 | 0.0.041 |
| (14-22-15*)l | 197 | 19 | 3.0.031 | 0.0.517 |
| (15-22-16*)l | 206 | 23 | 3.2.420 | 0.0.409 |
| (16-23-15*)l | 225 | 23 | 3.3.393 | 0.0.371 |
| (20-23-15*)l | 70 | 6 | 4.5.433 | 0.0.138 |
| (21-23-15*)l | 192 | 23 | 3.2.881 | 0.0.392 |
| (22-23-19*)l | 257 | 26 | 3.2.395 | 0.0.407 |
| (22-23-20*)l | 78 | 6 | 4.6.877 | 0.0.119 |
| (20-23-15*)r | 61 | 2 | 16.9.213 | 0.0.000 |
| **Nicotine only** | | | | |
| (6-7-27*)f | 270 | 52 | 3.6.597 | 0.0.268 |
| (14-22-23*)l | 251 | 45 | 5.5.246 | 0.0.044 |
| (21-23-14*)l | 230 | 41 | 3.7.411 | 0.0.250 |
| (22-23-14*)l | 249 | 46 | 4.0.703 | 0.0.181 |
| (22-23-16*)l | 264 | 46 | 3.5.680 | 0.0.294 |
| (15-22-21*)r | 189 | 33 | 3.4.359 | 0.0.339 |
| (22-23-21*)r | 244 | 43 | 3.2.816 | 0.0.390 |
| **Substances only** | | | | |
| (2-3-11*)f | 156 | 7 | 11.5.819 | 0.0.000 |
| (2-3-12*)f | 159 | 7 | 6.9.706 | 0.0.013 |
| (2-3-13*)f | 156 | 7 | 11.1.964 | 0.0.000 |
| **COMBINED EXPOSURES** | | | | |
| **Alcohol + Nicotine only** | | | | |
| (1-4-11*)f | 155 | 10 | 3.8.776 | 0.0.227 |
| (5-8-2*)f | 158 | 10 | 5.2.542 | 0.0.061 |
| (15-22-20*)l | 70 | 7 | 3.2.839 | 0.0.430 |
| (22-23-20*)l | 78 | 7 | 3.6.223 | 0.0.311 |
| (22-23-19*)r | 266 | 19 | 3.7.114 | 0.0.257 |
| **Alcohol + 1 Drug only** | | | | |
| (16-23-22*)l | 266 | 5 | 7.4.130 | 0.0.007 |
| (22-23-14*)l | 249 | 4 | 3.3.597 | 0.0.363 |
| (22-23-15*)l | 207 | 5 | 4.1.347 | 0.0.173 |
| (22-23-16*)l | 264 | 5 | 3.2.462 | 0.0.405 |
| (22-23-20*)l | 78 | 1 | 4.4.891 | 0.0.144 |
| (22-23-21*)l | 241 | 4 | 3.2.015 | 0.0.424 |
| (14-22-15*)r | 191 | 4 | 10.4.048 | 0.0.001 |
| (22-23-21*)r | 244 | 5 | 10.0.679 | 0.0.001 |
| **Alcohol + more than 1 Drugs only** | | | | |
| (21-23-14*)l | 230 | 2 | 3.1.498 | 0.0.447 |
| **Nicotine + 1 Drug only (n)** | | | | |
| (1-4-6*)f | 159 | 14 | 3.3.852 | 0.0.362 |
| (1-4-7*)f | 157 | 14 | 2.9.748 | 0.0.538 |
| (1-4-11*)f | 155 | 13 | 3.0.660 | 0.0.493 |
| (14-22-21*)r | 228 | 15 | 4.8.090 | 0.0.090 |
| (16-23-14*)r | 259 | 20 | 6.2.155 | 0.0.023 |
| (16-23-17*)r | 247 | 17 | 5.8.477 | 0.0.033 |
| **Nicotine + more than 1 Drug only** | | | | |
| (14-22-20*)l | 81 | 4 | 2.9.720 | 0.0.567 |

**Table 3.** (Continued)

| Facial landmarks | Control (n) | Test (n) | F statistic | P value |
|---|---|---|---|---|
| (15-22-16*)l | 206 | 5 | 4.0.671 | 0.0.185 |
| (15-22-23*)l | 208 | 5 | 2.8.789 | 0.0.584 |
| (16-23-15*)l | 225 | 6 | 4.3.017 | 0.0.147 |
| (19-23-15*)l | 212 | 6 | 3.1.237 | 0.0.460 |
| (20-23-14*)l | 81 | 3 | 5.5.724 | 0.0.054 |
| (21-23-14*)l | 230 | 7 | 4.0.852 | 0.0.180 |
| (21-23-15*)l | 192 | 6 | 3.2.418 | 0.0.412 |
| (22-23-15*)l | 207 | 5 | 3.0.311 | 0.0.504 |
| (14-22-16*)r | 249 | 7 | 4.7.735 | 0.0.092 |
| (20-23-14*)r | 67 | 2 | 3.2.537 | 0.0.449 |
| **Alcohol + Nicotine + 1 Drug only** | | | | |
| (17-23-15*)r | 198 | 10 | 3.4.657 | 0.0.331 |
| (20-23-15*)r | 61 | 3 | 8.7.389 | 0.0.005 |
| (22-23-17*)r | 235 | 9 | 2.4.352 | 0.0.897 |
| **Alcohol + Nicotine + more than 1 Drugs only** | | | | |
| (14-22-20*)l | 81 | 3 | 4.0.260 | 0.0.215 |
| (14-22-21*)l | 228 | 7 | 4.4.492 | 0.0.127 |
| (20-23-15*)l | 70 | 3 | 4.9.858 | 0.0.095 |
| (22-23-20*)l | 78 | 3 | 3.6.279 | 0.0.312 |
| (14-22-17*)r | 224 | 6 | 4.0.560 | 0.0.186 |
| (14-22-20*)r | 65 | 3 | 5.1.528 | 0.0.084 |
| (16-23-14*)r | 259 | 7 | 9.3.267 | 0.0.001 |
| (21-23-14*)r | 229 | 6 | 3.3.077 | 0.0.383 |
| (22-23-16*)r | 271 | 8 | 7.1.205 | 0.0.010 |
| (22-23-17*)r | 235 | 7 | 11.7.991 | 0.0.000 |
| (22-23-19*)r | 266 | 7 | 7.6.040 | 0.0.006 |

KEY: f = frontal landmark, l = left landmark, r = right landmark, * = apical landmark with respect to the base of the triangle, F statistic and p values are based on Hotelling's T2 analysis

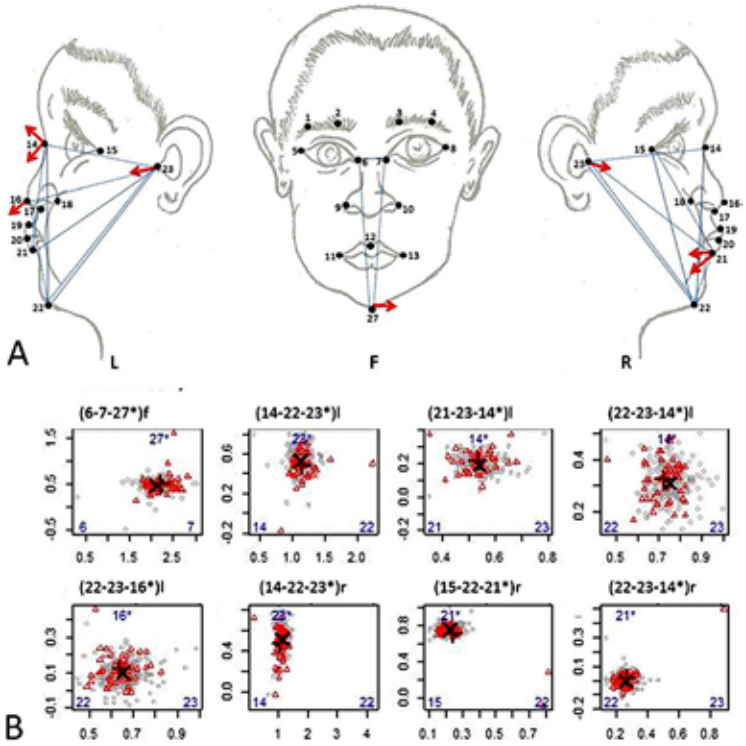


**Figure 3.** Facial landmark deviations in neonates exposed to only Nicotine. A) One frontal, three right and four left lateral triangles were significantly altered ($p \leq 0.0.5$). B) Hotelling's $T^2$ test discrimination of statistically significant triangle deviations between 'Nicotine only' and control group.

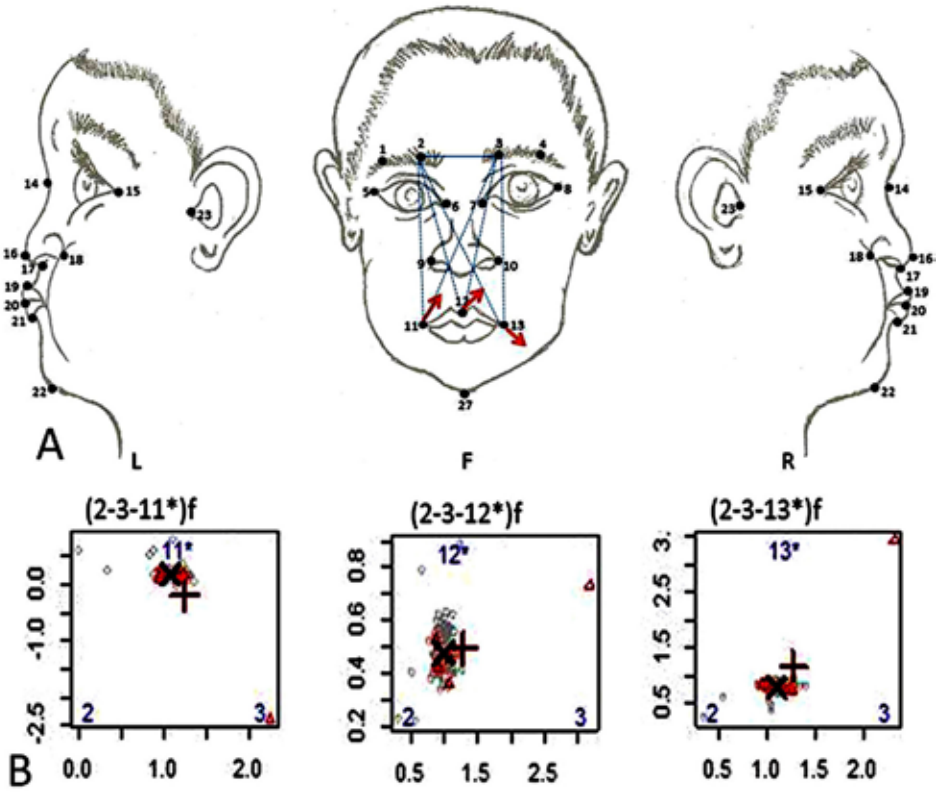


**Figure 4.** Facial landmark deviations in neonates exposed to only Drug. A) Only three frontal triangles were significantly altered (p≤0.0.5). B) Hotelling's $T^2$ test discrimination of statistically significant triangle deviations between 'Drugs only' and control group.

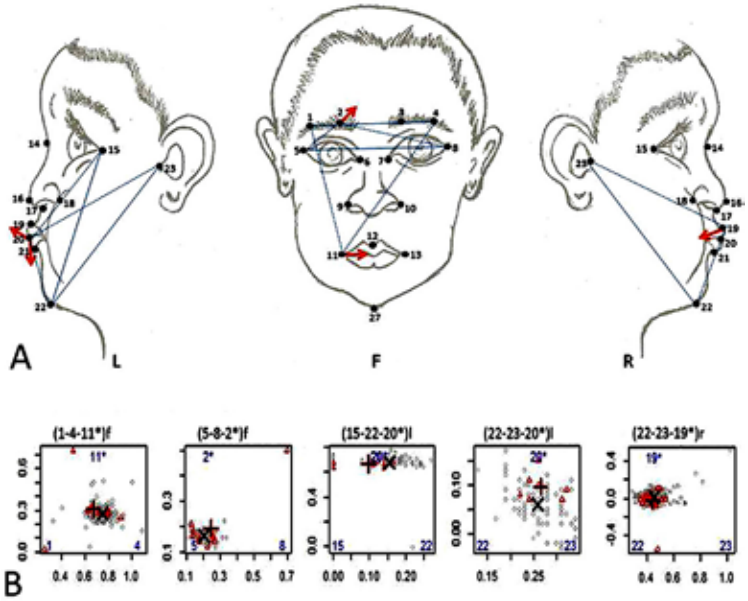


**Figure 5.** Facial landmark deviations in neonates exposed to Alcohol and Nicotine only. A) Two frontal, one right and two left lateral triangles were significantly altered (p ≤ 0.0.5). B) Hotelling's $T^2$ test discrimination of statistically significant triangle deviations between 'Alcohol and Nicotine only' and control group.

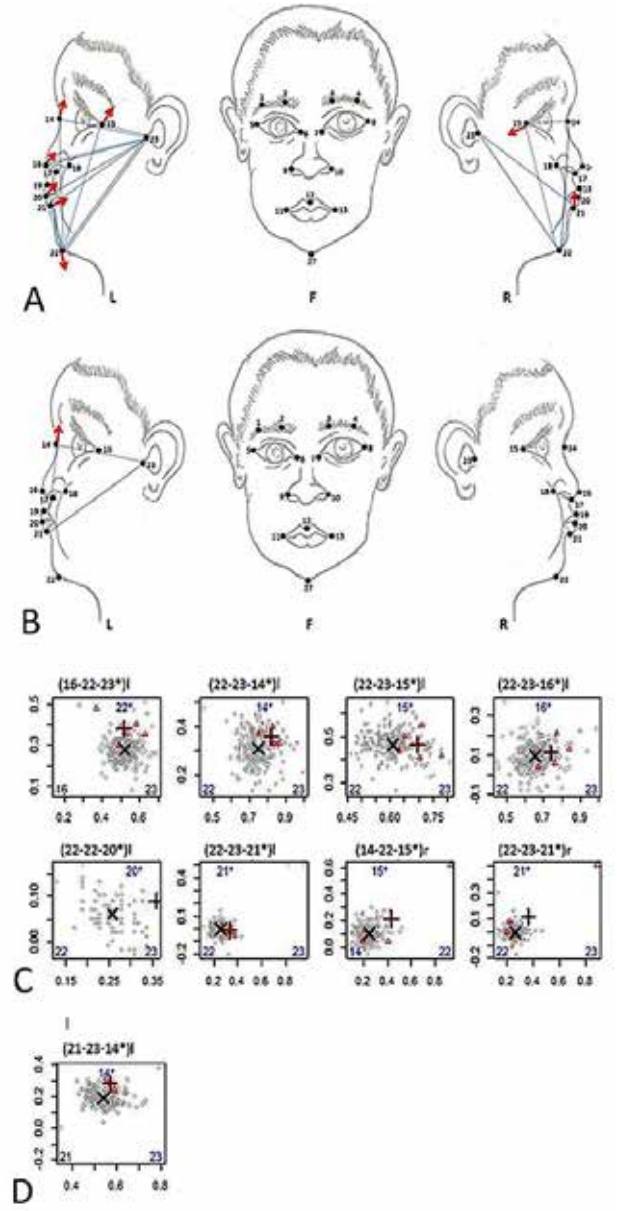


**Figure 6.** Facial landmark deviations in neonates exposed to Alcohol and drugs. A) Exposure to alcohol and one drug displayed two right and six left lateral triangles were significantly altered (p ≤0.0.5). B) Facial landmark deviations in neonates exposed to Alcohol and greater than one drug manifested only one left triangle was significant (p ≤0.0.5). C) Statistically significant triangle deviations between Alcohol and only one drug and control group. D) Statistically significant triangle deviations between alcohol and greater than one drug and control group.

triangle (6-7-27*)f was statistically significant in that the apical landmark 27 is at the lowest tip of the chin. These data suggested a lateral distortion of the chin and possibly increased canthion distance with respect to the entrocanthion. The distortion data of left lateral triangles showed protrusion and vertical lowering of the nasion and pronasale, downward slanting with reduced distance of the tragnion towards the exocanthion possibly causing flattened features, reduced distance between the tragnion and labiale inferious with respect to the lateral depth possibly causing larger lower lip.

### 4.1.3. Substance (s) exposure

In African-American neonates exposed to only substances *(*Figure 4), which included abuse of either one or a combination of the four main substances, (*i.e.* marijuana and cocaine), three statistically significant changes in frontal landmark triangles (2-3-11*)f, (2-3-12*)f, and (2-3-13*)f were observed. These results indicate a reduced length of the chelion with respect to the midface and labiale superious, and an overall slanting distortion of the mouth region.

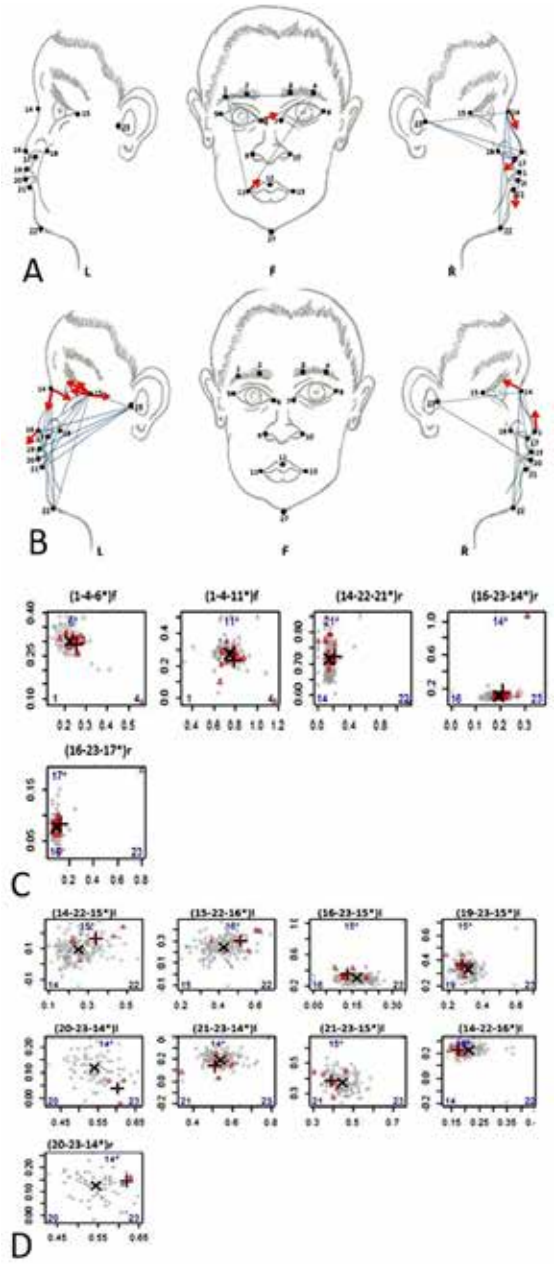


**Figure 7.** Facial landmark deviations in neonates exposed to Nicotine and Drugs. A) Exposure to nicotine and one drug shows evidence of two frontal and three right lateral triangles were significantly altered. B) Exposure to nicotine and greater than one drug shows evidence of two right lateral and seven left lateral triangles were significant (p≤0.0.5). C) Statistically significant triangle deviations between 'Nicotine and One Drug and D) nicotine plus greater than one drug.

### 4.1.4. Alcohol and nicotine combined exposure

African-American neonates exposed to alcohol and nicotine (Figure 5), showed reduced chelion distance and increased distance in the intersections of the eyebrow curve perpendicular to the palpebral fissures. These data indicate morphometric detectability of reduced interpupillary distance and lowered protruding or indistinct stomion with possibly increased concavity of the philtrum or thicker upper lip.

### 4.1.5. Alcohol and substance (s) combined exposure

Neonates exposed to alcohol and substances (Figure 6) were studied in two groups: 'Alcohol exposure with one substance' and those exposed to 'Alcohol and greater than one substance'. The data for Alcohol and only one substance group indicated an interesting pattern consistent with overall flattening of the face evidenced by a raised nasion and lateral shortening of the exocanthion towards the ear, inward

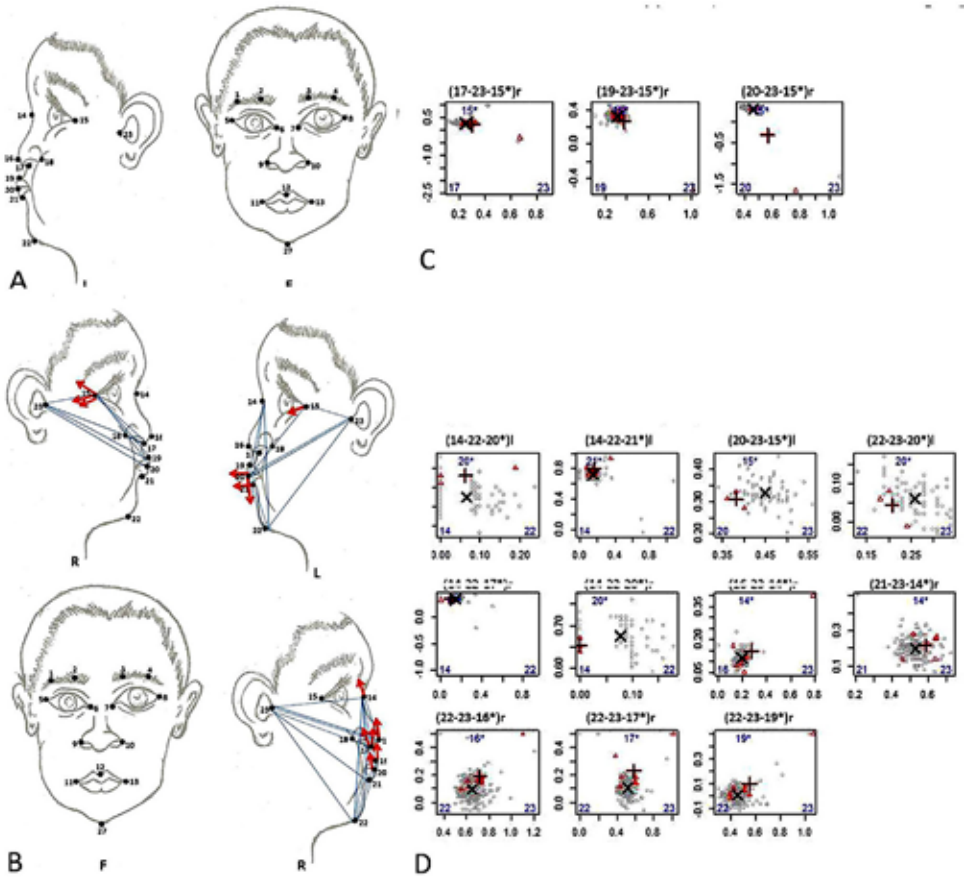


**Figure 8.** Facial landmark deviations in neonates exposed to Alcohol and Nicotine and Drugs. (A) Alcohol, nicotine and one drug shows evidence of three right lateral triangles significantly altered. B) Alcohol, nicotine and greater than one drug shows evidence of six right lateral and four left lateral triangles were significant (p≤0.0.5). C) Statistically significant triangle deviations between 'alcohol, nicotine and one drug group' vs control group and (D) greater than one drug vs control group.

shortening of the pronasale, stomion and labiale inferious associated with elongating the face through downward increase in distance to the gnathion. These landmarks were observed as significant lateral triangle distortions with no frontal dislocations. The alcohol and greater than one substance exposure group displayed only one significant triangle in the left lateral, which also showed a similar pattern of raised nasion height between the eyebrow curvatures. Overall, facial dissymmetry was observed for the left side of the face.

### 4.1.6. Nicotine and substance (s) combined exposure

Neonates exposed to nicotine and substances (Figure 7) were also studied in two groups: those exposed to 'nicotine and one substance' and 'nicotine and greater than one substance'. The group exposed to 'nicotine and one substance' showed increased entrocanthion distance, raised chelion on one side, lowered nasal bridge, protruding subnasale and lowered labiale inferious causing possible increased or protrusion of the lower lip. In the group exposed to 'Nicotine and greater than one substance', other lateral characteristics were distinguishable, suggestive of a lowered nasal bridge, reduced canthion distance and downward slanting of the eyes, raised pronasale and increased labiale superious, possibly increased vermillion length.

### 4.1.7. Alcohol, nicotine and substance (s) combined exposure

Neonates exposed to these combined substances *(*Figure 8), were studied in two groups: those exposed to 'alcohol, nicotine and one substance' and those exposed to 'alcohol, nicotine and greater than one substance'. The only notable landmark displacement in the 'alcohol, nicotine and one substance' exposed group was observed with respect to the lateral intersection of upper and lower eyelids suggesting lowered exocanthion indicative of downward slanting eyes and increased canthion length. Exposure to 'alcohol, nicotine and greater than one substance' showed lateral landmark features protrude with respect to stomion, labiale inferious. An upperward distortion of the labiale superious, subnasale, pronasale and higher nasion was recorded. In both categories, no frontal landmark

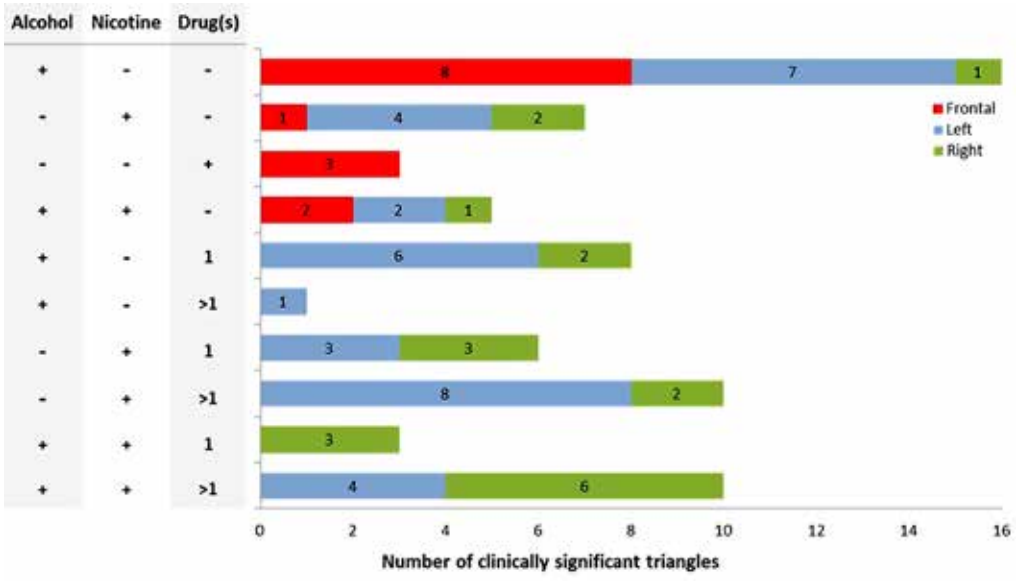


**Figure 9.** Frequency of statistically significant dislocated facial landmark triangles in exposure groups.

displacements were noted, but prominent upward directional asymmetry was observed for the right side of the face.

## 5. DISCUSSION

Clinicians using FASD diagnostic criteria routinely screen neonates exposed *in utero* to alcohol. Observing these facial features can potentially enable early interventions to cognitive impairment. The goal of this study was to investigate whether morphometric analysis of facial features can be applied as a postnatal clinical screening tool to identify neonates exposed to other teratogenic substances, and it can be used to predict neonates at risk for cognitive impairment. Geometric morphometrics was used to identify facial structure dislocations from key landmarks using photogrammetry as a simple tool for clinical execution. These investigations identified unique facial landmarks associated with various substance exposures. A major strength of this research model was to study the compounding effects of various teratogens. It recognizes subtle dysmorphic facial changes that could be challenging to the naked eyes of the clinician.

Overall, data from Figure 9 indicates that a greater number of clinically significant *facial landmark dislocations* were observed in the 'Alcohol only' group (16) as compared to any other single and/or combined teratogen group exposure (≤ 10). This suggests that neural crest migration and craniofacial development are more sensitive to the effects of alcohol during gestation. The clinical dislocations were consistent with the previously established FASD features (40, 50). In comparison, nicotine alone caused proportionally less *frontal landmark dislocations* and more lateral dislocations, whereas substances alone caused only midline alterations seen through *frontal landmark dislocations*.

Interestingly, when substance (s) exposures were combined with alcohol and/or nicotine, only lateral landmark dislocations were observed, but no *frontal landmark dislocations* noted (Figure 9). With respect to *frontal landmark dislocations*, the 'alcohol only' group caused eight *frontal landmark dislocations*, but interestingly no frontal dislocations were detected when alcohol was combined with other substances (*i.e.* cocaine, marijuana and/or heroin) or 'nicotine and substances'. These findings suggest that combined exposures do not appear to cause the same extent of aberrant midline neuronal crest migration. When alcohol is consumed along with nicotine, there was a marked decrease in frontal and lateral facial landmarks. These observations suggested that the effects of nicotine and substances counteract alcohol-mediated aberrant neuronal cell migration, thereby reversing the net vector change in triangle distortion. We postulate that nicotine and substances may promote localized effects on apoptosis, as well as on CNS cell migration and differentiation thus affecting alcohol's effect on neuronal cell migration.

We observed that certain landmark dislocations were associated with one class of teratogen, while a different set of landmark dislocations were observed for other teratogen (s). The combined effect of several teratogens showed subtractive or reduced landmark dislocations and at times unique landmark dislocations, this could be a consequence of specific teratogens mediating various effects on the molecular mechanisms determining the overall morphology of the face. A previous study conducted by Moore *et al.* in 2002 also confirmed that the effects of prenatal alcohol exposure

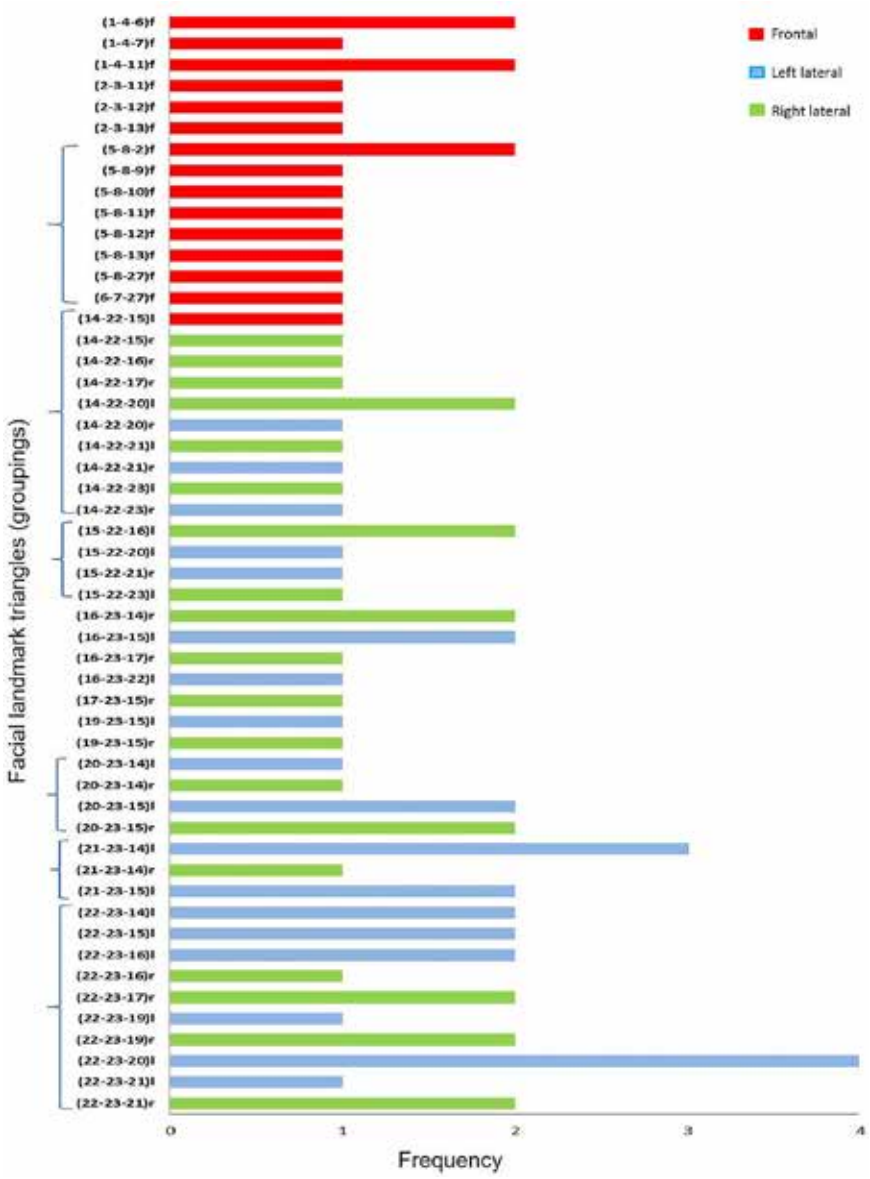


**Figure 10.** Frequency of exposure group mediated distortions of clinically significant facial landmark triangles.

on the face fell along a continuum instead of occurring as discrete traits, and that there appeared to be a range of quantitative facial effects (53). Establishing significantly specific dislocated landmarks in neonates could suggest specific exposure to physicians as predictors of developmental, neurological and cognitive concerns (53, 63).

Upon observing the detectable frequencies of all significantly dislocated triangles, we identified an association with three main bases. Base points 5-8, the distance between outer canthions, had seven significant triangles; base 14-22, the distance between the nasion and gnathion, had ten significant triangles and base 22-23, the distance between the tragnion and gnathion, had ten significant triangles (Figure 10). These results suggest that studying smaller triangles might be limiting to clinical relevance as a tool for diagnosis. The major informative axis of the frontal face is the horizontal distance between the canthions, while the major informative distance of the lateral face (left and/or right) lies between any combination of the distances between the nasion, gnathion and tragnion.

In this study, the analyses were limited to the effects of teratogen-induced dysmorphisms of the face based on maternal recall of her prenatal history. This was challenged by the inability to quantify, or investigate the timing of exposure as well as other biological systems (maternal medical conditions, family neurologic risk factors, medications, delivery risk factors, etc.) and other confounders of bioavailability.

However, this study is like other studies on prenatal substance exposures which relies on maternal history recall. The concurrence of toxicology testing remains challenging with still no gold standard of biologic sampling. Additionally, neonate anthropometric measures and gender were not standardized to deviations in landmark dislocations. Although there was a large sample size (Hotelling's $T^2$ test, validates $p \leq 0.0.5$), the categories of substance exposure were small when extracted. Our current data identified facial landmarks that were significantly altered by exposure to various substances. Our study noted that combined exposures were associated with reduced detectable changes than exposures to single agents.

The goal of this study was to assess a screening tool tailored to identify the effects of teratogen exposure using facial changes in our African- American neonatal cohorts. This tool is promising for differentiating morphometric facial changes that could predict physical, developmental and neurological problems associated with substance consumption during pregnancy. By analyzing clinically relevant landmark triangles of the face, a set of unique and significant dysmorphic features were obtained for certain teratogenic exposure. Morphometrics can be a valuable tool for screening neonates and possibly predicting other teratogenic exposure when there has been no underlying neurologic compromise at birth. This preliminary study provides justification for further studying the effects of prenatal substance exposure on the abnormal changes in facial development of the neonate immediately following birth.

In conclusion, this preliminary finding of prenatal exposure to substances, either alone or in combination, with alcohol exposure leads to amelioration of the alcohol-related facial features. Noting, a neonate who was prenatally exposed to alcohol but had an apparently normal facial appearance might not be followed as intensely postnatally. Therefore, clinicians need to be aware that if the mother consumed other substances, the standard FASD facial features may not be diagnostically relevant for determining clinical course of care based on assumed exposure. The use of morphometric analysis may have clinical value to the early neonate when toxicology results are misleading and subtle features are not evident.

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**Abbreviations**: FASD: fetal alcohol spectrum disorders; LSD: lysergic acid diethylamide; IUGR: intrauterine growth retardation; ROS: reactive-oxygen species; THC: δ-9-tetrahydrocannabinol

**Key Words:** African-American, Alcohol, Cocaine, Fetal alcohol syndrome, Illicit substances, Neonates, Nicotine, Marijuana, Morphometric analysis, Teratogen

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